Model Generation and Testing to Probe Neural Circuitry in the Cingulate Cortex of Postmortem Schizophrenic Brain

by Francine M. Benes

Abstract

In the past decade, there has been increased interest in whether discreet alterations of neural circuitry might play a role in the pathophysiology of schizophrenia. In the absence of a readily identifiable histopathology, a variety of sophisticated neurobiological approaches is being applied to the study of this disorder. In one series of investigations, subtle abnormalities have been detected in the anterior cingulate cortex–layer II (ACCx–II) of schizophrenia patients. One of these studies suggested a reduction of nonpyramidal neurons in schizophrenia patients, and it was postulated that this change could give rise to a relative increase of dopaminergic inputs to the remaining gamma-aminobutyric acid (GABA) cells. Although empiric evidence in support of this hypothesis was obtained, a subsequent post hoc analysis, described in this report, has suggested that this change could have occurred irrespective of whether GABA cells are reduced in number. A shift of cortical dopamine afferents from pyramidal to nonpyramidal neurons in ACCx–II seems to provide a more plausible explanation for such a "miswiring." These findings support critical use of model generation and testing as powerful tools for unraveling the nature of altered neural circuitry in postmortem schizophrenic brain.

Key words: Brain, cingulate cortex, circuitry.
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> It is not necessary that you be convinced of the truth of a particular hypothesis to justify devoting one's energies to testing it. It is enough that one regard it as worth testing and finding the tools to be adequate. [Seymour Kety 1959, p. 1596.]

In the past 10 years, there has been a marked increase of interest in how corticolimbic circuitry may be altered in schizophrenia. This focus comes from a series of histopathological studies reporting alterations in the arrangement (Kovelman and Scheibel 1984; Jakob and Beckmann 1986; Benes and Bird 1987) and decreases in the density (Benes et al. 1986, 1991*a*; Falkai and Bogerts 1986; Pakkenberg 1987; Falkai et al. 1988; Jeste and Lohr 1989) of neurons in several key corticolimbic regions of the schizophrenic brain. Although some quantitative studies have shown no difference (Benes et al. 1991*a*; Heckers et al. 1991; Pakkenberg 1993; Akbarian et al. 1995; Arnold et al. 1995) or, in one case (Selemon et al. 1995), increases of neuronal density in the schizophrenic brain, there is an emerging consensus that subtle morphological changes in the brain probably play a role in the pathophysiology of this disorder (Benes 1993*b*). Unlike other brain disorders, such as Alzheimer's disease and Huntington's chorea, in which classic histopathological features are readily identifiable, patients with schizophrenia have not been shown to have any characteristic, consistent, or specific changes at either the gross or the microscopic level (Benes 1995). This fact has made studying this disorder particularly difficult because the goal of such investigations is so elusive.

One approach to the study of the postmortem schizophrenic brain offers considerable potential for insight into its underlying pathophysiology: model generation and testing to probe neural circuitry in key corticolimbic regions such as the anterior cingulate cortex (ACCx) (Benes 1993*a*). Carefully devised hypothetical constructs can provide systematic guidance to neuroanatomical studies so that subtle alterations along complex neural circuits can be identified in patients with schizophrenia (Benes 1993*a*). A series of studies focused primarily on the ACCx region has detected a number of differences in schizophrenic brains, particularly in layer II (Benes 1995). As each of these studies yielded findings, the findings were

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incorporated into a working model circuit that has been actively used to generate testable questions concerning these and other changes that might play a role in the pathophysiology or treatment of schizophrenia. In the discussion that follows, these various findings in the ACCx–layer II (ACCx–II) region are described in relation to model generation and testing in probing neural circuitry in the schizophrenic brain.

The intent in presenting this working model is not to convince the reader that the postulates regarding the ACCx are correct, but rather to demonstrate more generally how new insights may be gained as to how neural circuitry is altered in patients with schizophrenia. As the studies described below unfold, the reader will see that some aspects of the working model for the ACCx region have received confirmation from followup investigations and others have not. Either way, the information obtained has been illuminating, and it is particularly the unconfirmed aspects that have suggested surprising possibilities, not only ways the circuit may be altered in schizophrenia patients, but also the possible mechanism(s) that may have been involved. The test of a good model is not whether it confirms a preexisting idea, but whether it guides the investigator to novel insights concerning the problem under investigation.

Postmortem Findings in ACCx–II of Schizophrenia Patients

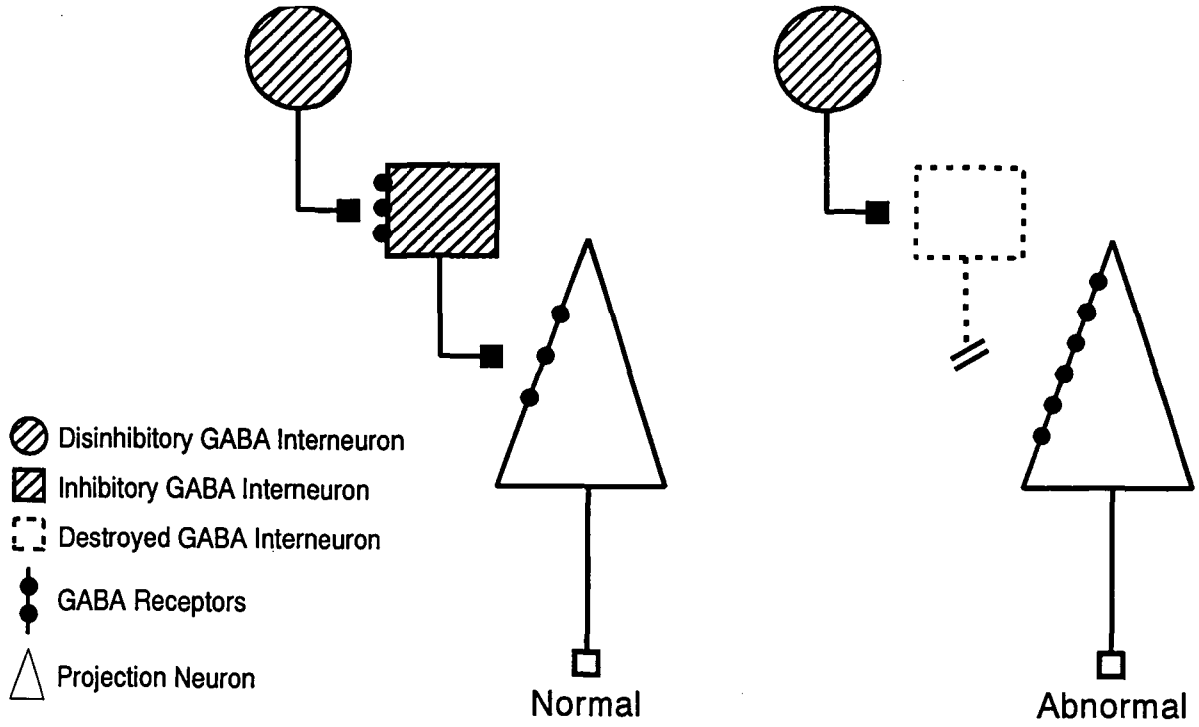
In 1972, a quantitative cell-counting study of a postmortem schizophrenic brain reported that there was a reduction in the density of neurons in the prefrontal (Brodmann area 10), ACCx (Brodmann area 24), and primary motor cortex (Brodmann area 4) (Colon 1971). Some years later, a second study using an entirely different counting technique also found a reduced density of neurons in these same three cytoarchitectonic regions (Benes et al. 1986). In the case of the second study, however, glial counts were also obtained and showed no difference in schizophrenia patients, suggesting that a typical degenerative process did not account for the differences in the patient group. It is noteworthy that this study, which employed transverse sections of the cortical gyri to minimize cytoarchitectural distortions, was validated in part by a normative investigation of human cortex in which tangential sections of Brodmann area 10, together with determinations of neuronal deoxyribonucleic acid (DNA) (Pope 1978), revealed strikingly similar cell densities. The remarkable correspondence of the first study with the second supported the idea that a reduced density of neurons in the cortex of schizophrenia patients might be both reliable and meaningful.

In a subsequent replicative study in which similar cell counting data were broken down according to pyramidal and nonpyramidal neuron subtypes (figure 1), it was found that schizophrenia patients showed a preferential reduction in the density of interneurons, particularly in ACCx–II (Benes et al. 1991*a*). Closer scrutiny of the data, however, revealed that the schizophrenia patients who had superimposed mood disturbances showed more prominent reductions in NP cell density than did the patients without affective symptoms, and suggested that this finding might show a stronger covariation with mood disorder. Despite this unexpected difference between the two subtypes of chronic psychosis, a working model was developed for ACCx in which a loss of inhibitory interneurons or activity was conceptualized as playing a role in both (Benes et al. 1989). Since gamma-aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the cortex (Jones 1987), it was postulated (figure 2), that a compensatory up-regulation of the $GABA_A$ receptor might be found on postsynaptic pyramidal neurons of the cortex of a schizophrenic brain (Benes et al. 1989).

To test this hypothesis, a high resolution technique for localizing $GABA_A$ receptor binding activity (Benes et al. 1989) was applied to a cohort of normal controls and schizophrenia patients and blindly analyzed by a computer-assisted quantitative technique. The results indicated a marked increase of this specific receptor activity in layers II and III, but not V and VI, of ACCx (Benes et al. 1992*b*). Similar findings have also been observed in the prefrontal cortex (Benes et al. 1996*b*) and hippocampal formation (Benes et al. 1996*a*) of schizophrenic brains. In the prefrontal area, like the ACCx, the findings were most marked in layer II (Benes et al. 1996*b*), where a reduction in the density of nonpyramidal neurons had previously been observed in both regions (Benes et al. 1991*a*) (see figure 3). In contrast to the $GABA_A$ receptor, an up-regulation of the benzodiazepine receptor has not been found in the brains of schizophrenia patients (Squires et al. 1993), but recent evidence suggests that there may be an uncoupling in the regulation of these two receptor subtypes in the schizophrenic brains (Benes et al. 1997*b*). Interestingly, the number of cells expressing messenger ribonucleic acid (mRNA) for glutamate decarboxylase (GAD), the enzyme that synthesizes GABA, is also decreased in the prefrontal cortex (Akbarian et al. 1995), with the changes being most marked in layer II. Thus, it appears that a decrease of GABAergic activity in the prefrontal and cingulate cortices may potentially play a unique role in the pathophysiology of schizophrenia.

The combined cell counting and $GABA_A$ receptor binding data, and more recently the in situ hybridization studies of GAD mRNA, have provided growing support for the hypothesis that schizophrenia, and possibly also

Figure 1. Compensatory up-regulation of gamma-aminobutyric acid $(GABA)_A$ receptor binding activity on postsynaptic pyramidal neurons (PNs) in response to a decrease in the number or activity of GABAergic interneurons in the anterior cingulate cortex (ACCx) of schizophrenia patients

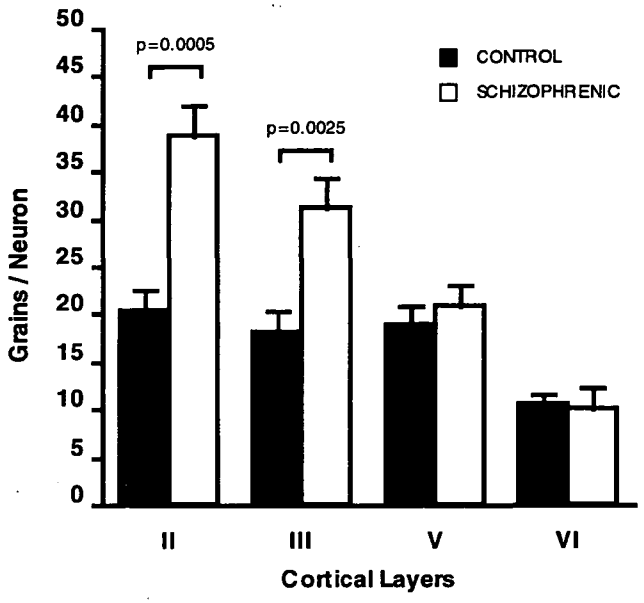


In the normal circuit, an inhibitory GABAergic cell provides input to the PN, which has GABA receptors (black balls) located on its somal membrane. The GABA cell itself also receives a GABAergic input from a "disinhibitory" cell and, accordingly, it too has GABA receptors located on its surface membrane. In the schizophrenia circuit, the loss of the inhibitory GABAergic neuron results in a compensatory up-regulation of the $GABA_A$ receptor on the PN. Overall, there is no net change in the amount of $GABA_A$ receptor binding in the larger circuit, making it necessary to use a high-resolution technique to analyze it on individual PNs. Adapted from Benes et al. 1989.

affective disorder, might involve a defect of GABA neurotransmission. This idea is not a new one, but was first suggested by Eugene Roberts as early as 1972. Corroborative neurochemical support for this hypothesis has come from various studies of the frontal cortex of the schizophrenic brain showing a decrease of GABA concentrations (Perry et al. 1979), a reduction in the specific activity of GAD (Bird et al. 1977), a decrease of GABA uptake (Simpson et al. 1989), and an increase of bicuculline-sensitive [^{3}H]muscimol binding (Hanada et al. 1987). The hippocampal formation of the schizophrenic brain also shows a decrease of GABA uptake (Reynolds et al. 1990) and an increase of $GABA_A$ receptor binding (Benes et al. 1996*a*). The fact that there is no change in benzodiazepine receptor binding in this region suggests that an uncoupling in the regulation of the $GABA_A$ complex may play a role in this disorder (Benes et al. 1997*b*). Although neurochemical data for other brain regions are not available, a reduction in the density of interneurons has also been observed in the dorsomedial nucleus of the thalamus (Dom 1976), suggesting that a defect in GABAergic activity may occur there as well.

The mechanisms responsible for the putative loss of GABAergic neurons or activity in schizophrenia are not known, but it has been suggested that a subtle excitotoxic process could potentially account for such changes and not require the presence of overt cell death (Benes 1993*c*; Olney and Farber 1995; Benes 1996*a*, 1996*b*). This idea has received support from two separate studies suggesting that schizophrenia patients have an increase of glutamatergic afferents projecting to superficial layers of ACCx. One study found an increase in the density of vertical axons visualized with antibodies against neurofilament protein (NFP–200K) (Benes et al. 1987). In the other study, fibers with a small caliber that were glutamate-immunoreactive (Benes et al. 1992*a*) showed an increase in ACCx–II in schizophrenia patients. These axons could potentially contribute to an excitotoxic effect on GABAergic interneurons in this lamina (figure 3). Some investigators believe that such an injury could involve a complex circuit in which the phencyclidine receptor contributes to a hypofunctioning of *N*-methyl-D-aspartate (NMDA)-sensitive glutamate receptors (Olney and Farber 1995). This kind of injury could involve either

Figure 2. Specific gamma-aminobutyric acid$_A$ receptor binding activity on neuronal cell bodies in layers II, III, V, and VI of the anterior cingulate cortex from normal controls (solid bars) versus schizophrenia patients (open bars)



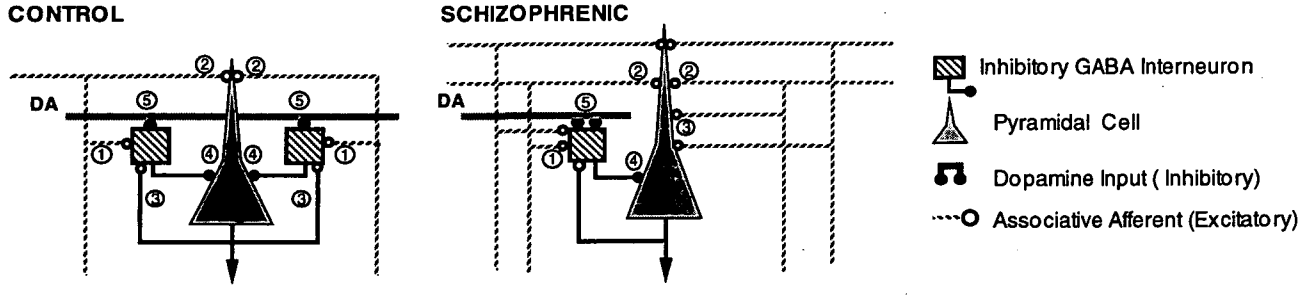
There is a marked increase of binding in layers II and III, but not V and VI, of the schizophrenia group. Adapted from Benes et al. 1992*b*.

cell loss or possibly a more subtle change at the cellular level with no overt loss of neurons. It could be exerted early in life (Farber et al. 1995), perhaps during the perinatal period, but it also could occur much later, during the postnatal period (Benes et al. 1996*c*) when dopamine (DA) fibers are attaining a maximal interaction with GABAergic cells (Benes 1988, 1993*a*, 1995). In fact, such changes may occur in relation to the prodrome of schizophrenia, which typically occurs during late adolescence and early adulthood (Kraepelin 1919/1921).

Testing the Model

DA-GABA Interactions in Relation to Schizophrenia. A model that seeks to describe neural circuitry changes in schizophrenia must incorporate the DA system because it is well known that typical neuroleptic drugs are selective antagonists of D_2 receptors, even though atypical drugs such as clozapine, while less selective, do show moderate affinity for both D_1 and D_2 receptor subtypes (Meltzer et al. 1989). Accordingly, the model depicted in figure 3 also incorporates DA projections to ACCx–II and postulates that, in schizophrenia patients, these afferents may be increased with respect to GABAergic interneurons. Although it was at one time generally believed that DA fibers in cortex interacted almost exclusively with pyramidal neurons, recent double immunocytochemical studies

Figure 3. Model depicting possible changes in the neural circuitry of layer II in the anterior cingulate cortex of patients with schizophrenia



In the control circuit (left), there are two glutamatergic excitatory inputs projecting to two gamma-aminobutyric acid (GABA)ergic interneurons (1) and the apical dendrite of the pyramidal neuron (2), which also sends an excitatory input to the inhibitory GABA cell (3). The GABA cells send an inhibitory input directly to the cell body of the pyramidal neuron (4), but themselves receive one inhibitory input from dopaminergic afferents (5). In the schizophrenia circuit (right), four glutamatergic afferents provide an excitatory input to one GABAergic interneuron (1) and the apical dendrite of the pyramidal neuron (2). The pyramidal neuron sends a recurrent collateral excitatory input to the GABAergic cell (3), which in turn sends a diminished GABAergic input to the pyramidal cell (4). The GABA cell is depicted as receiving two, rather than one, dopaminergic inputs as a result of a possible loss of the other interneuron (5). Taken together, this model predicts that a loss of GABAergic cells or activity would result in a decreased inhibitory modulation of the pyramidal neuron. An increase of inhibitory dopaminergic inputs to the GABA cell could further compromise the integrity of the circuit by further diminishing the inhibitory outflow of GABAergic activity toward the pyramidal cell. The increase of excitatory inputs entering the anterior cingulate cortex from other regions would markedly exacerbate the balance in this circuit and could itself be a proximate cause of excitotoxic damage to the GABAergic interneurons. Adapted from Benes 1993*c*.

conducted at both the light (Benes et al. 1993) and the electron (Verney et al. 1991) microscopic levels have demonstrated that contacts between dopamine fibers and GABA-immunoreactive neuron somata do occur. At the ultrastructural level, they appear to account for only 5 percent of all such terminals (Verney et al. 1991), but at the light microscope level, they appear to be much more common (Benes et al. 1993). While it is likely that these contacts are nonsynaptic in nature (Seguela et al. 1988; Verney et al. 1991), they occur frequently and show a nonrandom distribution (Benes et al. 1993). It is important to point out, however, that the light microscopic approach employed in this double immunofluorescence study had limited spatial resolution, and such contacts, though frequent, can only be considered putative in nature. Support for the notion that these contacts are functional, however, has come from studies showing both D_1 and, to a lesser extent, D_2 receptor binding activity associated with interneurons in the medial prefrontal cortex of rats (Vincent et al. 1993, 1995). These findings are consistent with data from an in situ hybridization study showing both D_1 and D_2 receptor mRNA localized in both pyramidal and nonpyramidal neurons somata (Huntley et al. 1992).

Since DA is believed to be an inhibitory neuromodulator, the model shown in figure 4 predicts that blockade of its receptors on nonpyramidal neurons could result in an increase of GABAergic effects being exerted on pyramidal neurons. It is important to emphasize that the interaction of DA afferents with intrinsic cortical elements is probably far more complex than figure 3 depicts. For example, both the D_1 agonist SKF38393 and the D_2 agonist RU24926 have been found to inhibit the electrically evoked release of ^{3}H-GABA in the medial prefrontal cortex of rats (Penit-Soria et al. 1989; Retaux et al. 1991*a*, 1991*b*). Paradoxically, D_2 agonists have been found to increase the spontaneous release of this transmitter. This apparent inconsistency could be explained by an initial depolarization induced by the action of DA on the D_2 receptor leading to either a presynaptic inhibition or a refractory period in GABA cells (Retaux et al. 1991*a*). Because D_2 receptors are mainly associated with inhibitory mechanisms, the complexity of these interactions between DA terminals and GABA cells in prefrontal cortex might well mask a similar effect on evoked GABA release. Specifically, DA binding to D_2 receptors on some terminals could produce an inhibition of GABA release, which in turn results in a disinhibition of a GABA neuron (Retaux et al. 1991*a*). It also seems plausible that the paradoxical effects of DA agonists could potentially be explained by an interaction of these agents with both inhibitory and disinhibitory GABAergic elements. Such an arrangement could help to explain the opposite effects observed for spontaneous versus evoked release of GABA in the medial prefrontal cortex. These issues are made even more confusing by a study reporting that DA elicits excitatory potentials in GABAergic neurons of pyriform cortex (Gellman and Aghajanian 1993). However, this region is a primitive allocortex with a simple three-layered structure, and its intrinsic circuitry may not be representative of that found in neocortical areas, such as medial prefrontal cortex.

To test the hypothesis presented in figure 4, rats were treated for 16 weeks with the typical neuroleptic, haloperidol (1 mg/kg/day), and a quantitative analysis of GABA–IR (immunoreactive) terminals surrounding pyramidal neurons was undertaken (Vincent et al. 1994). As predicted, there was a significant increase of GABA terminals on pyramidal neurons in the drug-treated group, a finding that is consistent with the idea that DA is inhibitory to GABA cells and that the interaction between these two systems could play at least a partial role in the pathophysiology or treatment of schizophrenia.

Is the Cortical DA Innervation Abnormal in Schizophrenia? A pharmacological agent can relieve the symptoms of an illness by either direct or indirect effects on a particular neurotransmitter system, such as that for GABA. As suggested by the model in figure 3, it was critical to obtain evidence for an increase of DA inputs to interneurons in ACCx–II. For this purpose, it was not possible to employ the same antibodies against DA that were used in the haloperidol study described above, because this low molecular weight soluble transmitter rapidly diffuses away from its storage sites within the tissue and cannot be reliably visualized in human postmortem cortex with postmortem intervals. An alternative strategy for testing the model was the localization of tyrosine hydroxylase (TH), the enzyme responsible for the synthesis of catecholamines. Since TH is a marker for both dopaminergic and noradrenergic fibers, its localization cannot be used to make specific inferences about one or the other transmitter system, even though most fibers visualized with this antibody are believed to be dopaminergic in nature (Lewis et al. 1987; Gaspar et al. 1989; Noack and Lewis 1989; Samson et al. 1990; Akil and Lewis 1993; Williams and Goldman-Rakic 1993). Moreover, the fact that a monoclonal antibody against TH was capable of producing an extensive and reliable localization of this marker in human postmortem tissues significantly offsets its relative lack of specificity for the DA system.

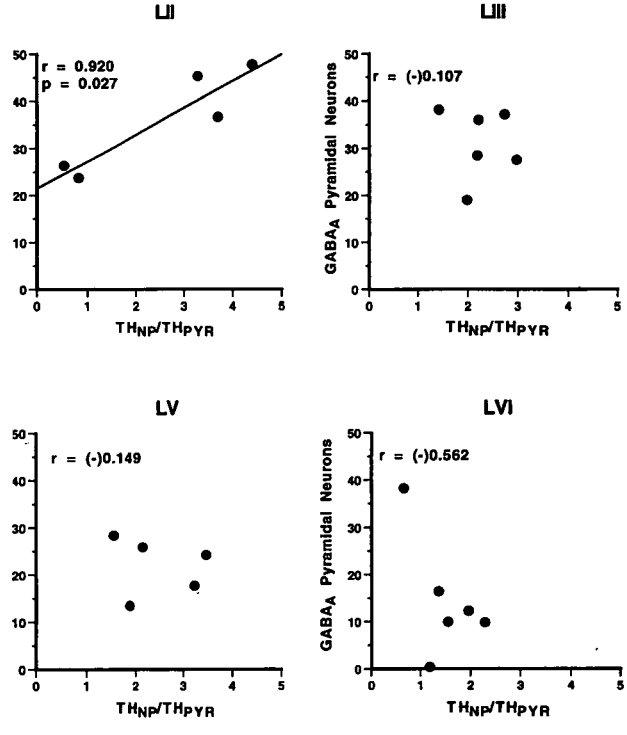
When the distribution of tyrosine-hydroxylase-immunoreactive (TH–IR) fibers was blindly analyzed in a cohort of control subjects and schizophrenia patients, the data showed no difference in the density of varicosities on pyramidal or nonpyramidal neurons, or in neuropil of ACCx or prefrontal cortex of the two groups (Benes et al.

1997*a*). When the identical data were expressed in an alternative fashion, however, a potentially important pattern presented itself. As shown in figure 4, the data for the density of TH–IR varicosities in contact with pyramidal versus nonpyramidal neurons were separately represented for controls (upper panel) and schizophrenia patients (lower panel). Rather surprisingly, the control subjects had a marked tendency for nonpyramidal neurons to show a higher density of contacts than pyramidal neurons, particularly in layers III and V, where the differences were significant. A similar pattern was also observed for the schizophrenia patients, although in their case the differences were even more marked, particularly in layer II, where the density of TH–IR varicosities was three times higher on nonpyramidal neurons. In layer II of the control subjects, no difference between pyramidal and nonpyramidal neurons was observed. Overall, these data are consistent with the model presented in figure 3, except that they now emphasize the fact that DA fibers interact extensively with the dendrites (Goldman-Rakic et al. 1989) and somata (Benes et al. 1993) of pyramidal neurons. Specifically, these most recent data suggest that there may be a small, but significant, decrease of catecholaminergic inputs to pyramidal neurons in ACCx–II of schizophrenia patients.

This finding is consistent with the results of another study in which TH–IR fibers have been found to be decreased in the neuropil of the entorhinal cortex in schizophrenia patients (Akil and Lewis 1995), where DA fibers may engage principally in synaptic connections with pyramidal cell dendrites (Goldman-Rakic et al. 1989; Verney et al. 1991). When the data for pyramidal neurons in the study of ACCx–II are considered in relation to nonpyramidal neurons, they are most consistent with a shift of DA fibers from projection cell bodies to interneurons, accounting for the apparent increase of these inputs to nonpyramidal neurons. It is theoretically possible that such a shift could also involve DA fibers that form synaptic connections with distal dendritic branches of pyramidal neurons; however, no change in the density of TH–IR varicosities was observed in the neuropil of ACCx in the schizophrenia group. While pyramidal and nonpyramidal neurons show a similar density of TH–IR fibers in the control group, their difference in relation to one another in the schizophrenia group is striking and suggests the possibility that this disorder may involve a miswiring of DA inputs in ACCx–II.

Use of Prediction in Critically Appraising a Neural Circuitry Model of Schizophrenia. An important criterion in evaluating the validity of a model is whether it is useful in making predictions (Henn and McKinney 1987). In the case of the working model shown in figure 3, an increase of inhibitory DA inputs could theoretically result in a decreased inhibitory modulation of pyramidal neurons by GABAergic cells. In the setting of a decrease in either the activity, or possibly the numbers, of these cells, an increased inhibitory influence of DA fibers on nonpyramidal neurons could result in a further reduction in the flow of GABAergic inhibition toward pyramidal neurons. If this assumption is correct, a significant covariation between the density of TH–IR varicosities and $GABA_A$

Figure 4. Preliminary data for the relationship between the ratio of the density of tyrosine hydroxylase-immunoreactive (TH–IR) fiber contacts on nonpyramidal versus pyramidal neurons (TH_{np}/TH_{pyr}) in relation to specific gamma-aminobutyric acid $(GABA)_A$ receptor binding activity in layers (L) II, III, V, and VI of the anterior cingulate cortex of normal controls and schizophrenia patients



The five cases included one control subject and four schizophrenia patients for whom both data sets were available. In layer II, there is a striking linear relationship between TH_{np}/TH_{pyr} and $GABA_A$ receptor binding activity ($r = 0.92$, $p = 0.027$). The data for the control case are represented at the lowest point along the regression line, while those for two neuroleptic-free schizophrenia patients were among the highest points. No linear relationship of this type was observed in layers III, V, and VI. The slope of the regression line is consistent with the hypothesis that an increase of inhibitory dopaminergic inputs to GABAergic cells could contribute to a compensatory increase of $GABA_A$ receptor binding activity on postsynaptic pyramidal neurons in layer II, where both data sets showed the most striking changes.

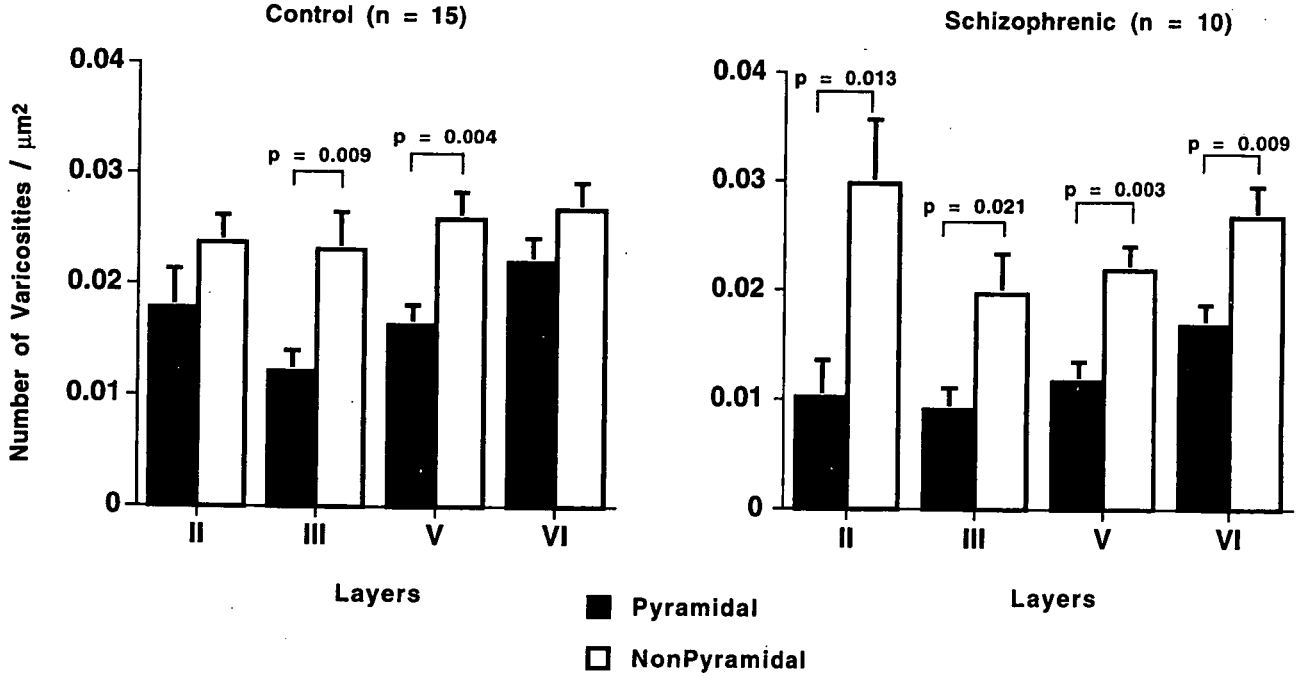
receptor binding activity might be expected to occur. To consider this possibility, as shown in figure 4, the density of TH–IR varicosities on nonpyramidal neurons was expressed as a ratio with respect to their density on pyramidal neurons (TH_{np}/TH_{pn}) and used as an independent variable; $GABA_A$ receptor binding activity was used as a dependent variable. As the model predicted, there is a very high correlation between these two variables in layer II ($r = 0.92$, $p = 0.027$), but not in layers III, V, and VI of ACCx in the five cases for which both data sets are available. Although there was only one control represented in the analysis, it is noteworthy that the data for this case fell at the lowest point along the regression line. In contrast, the data for two neuroleptic-free schizophrenia patients, also represented among the five cases, occurred at the highest point along the line. Thus, this preliminary analysis supports the idea that an increase of catecholaminergic inputs (presumptive DA afferents) to nonpyramidal neurons in ACCx–II might be related in some way to the up-regulation of the $GABA_A$ receptor observed in schizophrenia patients. This finding is also consistent with the idea that the increase of this binding activity on pyramidal neurons may represent a compensatory up-regulation of this receptor. It is obvious that such a conclusion is only inferential and that other possible explanations exist. For example, it is equally possible, until proven otherwise, that the increase of $GABA_A$ receptor binding activity in ACCx–II is completely unrelated to changes in the cellular distribution of TH–IR fibers, and the significant correlation represents only a grouping effect in control subjects versus schizophrenia patients. The magnitude of the covariation between the two variables ($r = 0.92$), however, does argue against this possibility.

A second critical use of the model presented in figure 3 is to make systematic predictions about putative relationships between dopamine fibers and various neuronal subtypes in ACCx of the schizophrenic brain. As shown in figure 6, there are three potential ways to conceptualize the finding of an increased density of TH–IR varicosities in contact with nonpyramidal neurons in layer II of ACCx of schizophrenia patients. For this purpose, the data obtained from the blind analysis of TH–IR varicosities were normalized using the empirically determined size of pyramidal and nonpyramidal neurons in layer II of this region. On average, pyramidal neurons were approximately two times larger in size than nonpyramidal neurons. Based on this observation, the size of pyramidal neurons was normalized to a value of 2.0, and that for nonpyramidal neurons was set at 1.0. Since there was no difference in the size of either cell type in the control subjects versus the schizophrenia patients, the same normalized values were used to express cell size in the patients with schizophrenia. In the control group, the density of TH–IR varicosities on pyramidal neurons was approximately equal to that of nonpyramidal neurons (figure 5). Accordingly, if the density of TH–IR varicosities was the same for both cell types in layer II of the controls (figure 5), then the number of such fibers forming contacts with pyramidal neurons can be represented as two per cell, and the number for nonpyramidal neurons as one per cell for the control subjects. Thus, regardless of cell type, the overall ratio of the density of TH–IR in contact with nonpyramidal versus that on pyramidal neurons in the control group is 1.0, a number that is almost identical to the one actually observed. The analogous ratio for the schizophrenia group is 3.0.

A ratio of 3.0 for the schizophrenia group could theoretically be obtained in three ways. First, if it is assumed that there is GABA cell loss in the schizophrenia patients, and that the TH varicosity from the lost cell forms a redundant contact with the surviving GABA cell (figure 6a), the resulting ratio of TH–IR varicosities on nonpyramidal versus pyramidal neurons for the schizophrenia group would be 2.0, a number that is lower than the observed ratio of 3.0. The second possible way to attain a higher ratio in the schizophrenia patients (figure 6b) would again assume that GABA cell loss occurs and that a TH varicosity moves to the surviving cell, but it would also assume a trophic shift of a TH varicosity from the pyramidal to the nonpyramidal neurons. In this case, the resulting ratio in schizophrenia patients would be 6.0, and, while it is higher than the 2.0 obtained with GABA cell loss alone (figure 6a), this number is much higher than the empirically observed ratio of 3.0. Finally, if it is assumed that there is no GABA cell loss, but only a trophic shift of a TH varicosity from the pyramidal to one of the nonpyramidal neurons (figure 6c), the ratio computed is 3.0, a number virtually identical to the one empirically determined from the data set. It is noteworthy that this last model also predicts that some nonpyramidal neurons would have double the normal number of TH varicosities, while others would essentially have a normal number. This pattern could explain the relatively high variance for the average density of TH–IR varicosities on nonpyramidal neurons observed in layer II of the schizophrenia group (figure 6*a*, *b*, *c*).

Based on the discussion above, a significant question is whether GABA cell loss contributed to the increase of TH–IR varicosities on nonpyramidal neurons in ACCx–II. To explore this possibility, another post hoc analysis was performed to determine whether there is evidence of a decrease in the density of nonpyramidal neurons in the sample fields of the schizophrenia patients versus control subjects that were obtained for the TH study. As shown in table 1, the density of pyramidal neurons was found to be 24.8 percent lower in the schizophrenia patients, while

Figure 5. Comparison of the density of tyrosine hydroxylase–immunoreactive (TH–IR) fiber contacts on pyramidal (PNs) versus nonpyramidal (NPs) neurons in various layers of the anterior cingulate cortex of controls and schizophrenia patients



In the control group (upper panel), there is a strong tendency for the density of TH–IR varicosities to be higher on NPs than on PNs, particularly in layers III and V, where the differences are quite significant. In the schizophrenia group (lower panel), a similar but more marked pattern can be observed, particularly in layer II where NPs have a density of TH–IR varicosities three times higher than on PNs. Adapted from Benes et al. 1996*b*.

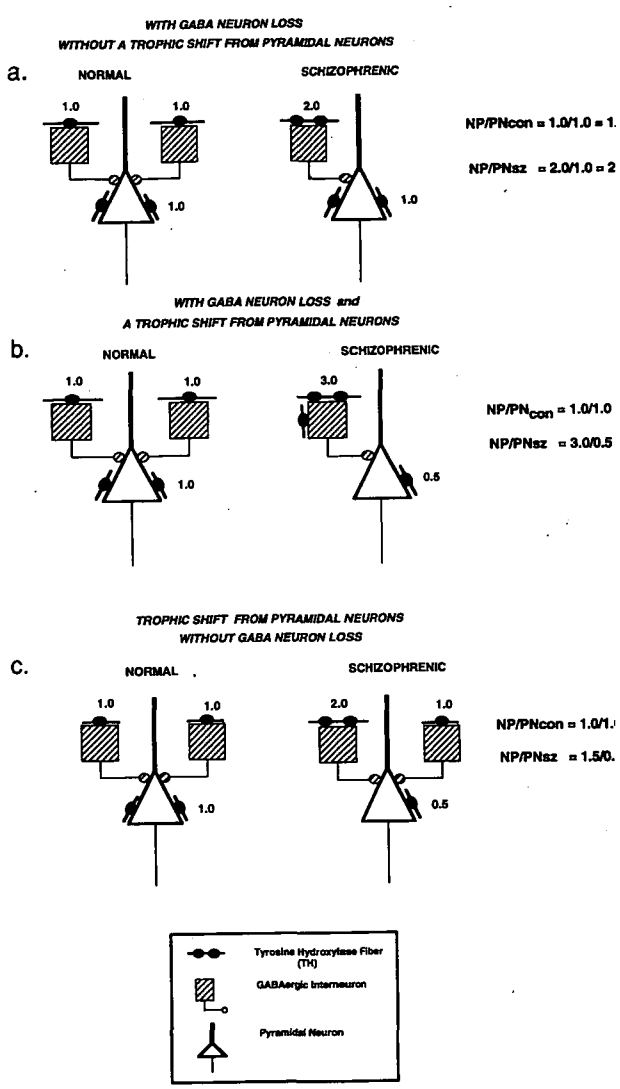
that for nonpyramidal neurons was 18 percent lower. It is important to emphasize that the sections employed in this study were 10 μm thick (Benes et al. 1997*a*), while those in an earlier cell counting study were 20 μm thick (Benes et al. 1991*a*). Since the average diameter of nonpyramidal neurons in layer II has been found to be approximately 9 μm, and that for pyramidal neurons approximately 18 μm, the sensitivity for detecting interneurons in a 10 μm thick section is intrinsically lower for nonpyramidal than for the larger pyramidal neurons (Wiebel 1979*a*, 1979*b*). Thus, the data for the density of nonpyramidal neurons obtained post hoc in the TH study (Benes et al. 1997*a*) are not as accurate as those reported in the earlier cell counting study (Benes et al. 1991*b*). As discussed above, it is noteworthy that schizophrenia patients without superimposed mood disturbance did not previously show dramatic reductions in the density of nonpyramidal neurons, while those with mood disturbance did (Benes et al. 1991*b*). Preliminary data from a more recent cell counting study are again suggesting that a reduction of nonpyramidal neurons may indeed show a stronger covariation with affective disorder than with schizophrenia (Vincent et al. 1997). Since the cohort included in the analysis of TH–IR varicosities was comprised of schizophrenia patients without superimposed mood disturbance, it seems plausible that a reduction of nonpyramidal neuron density may not have been present to a significant degree in this group. Thus, it is unclear at present whether nonpyramidal neuron loss plays a significant role in the distribution of TH–IR fibers in ACCx–II, and further study is needed to assess this possibility.

Table 1. A comparison of neuronal density in layer II of the anterior cingulate region of normal and schizophrenia patients

	Neuronal cell density (number/μm²)	
	Pyramidal cells	Nonpyramidal cells
Control group	10.9 ± 1.6	18.9 ± 1.7
Schizophrenia group	8.2 ± 2.1	15.5 ± 2.3
Difference (%)	24.8	18.0

Note.—The data shown are from the sample fields used in the analysis of tyrosine hydroxylase-immunoreactive (TH–IR) and are expressed as the "mean of means" ± standard error of the mean (SEM) for the individual cases in the control and schizophrenia groups.

Figure 6. Three model circuits used to consider how the observed increase of tyrosine hydroxylase–immunoreactive (TH–IR) varicosities on nonpyramidal neurons (NPs) in layer II of the anterior cingulate cortex of schizophrenia patients (see figure 4) could have occurred



The size of pyramidal (PNs) and nonpyramidal (NPs) neurons was normalized according to the empirically derived cell areas and the number of TH–IR varicosities per cell adjusted in proportion to the values determined. The three mechanisms considered in the diagram are (a) gamma-aminobutyric acid (GABA) neuron loss alone, (b) GABA neuron loss together with a trophic shift of TH varicosities from PNs to NPs, and (c) a trophic shift of TH varicosities from PNs to NPs with no loss of GABA cells. A ratio of the density of TH–IR varicosities on NPs versus PNs (NP/PN_{con} vs. NP/PN_{sz}) was computed for the control and schizophrenia groups, respectively, using each model. These numbers were compared to those empirically observed in the data shown in figure 4. Only the last potential model yields a theoretical ratio (3.0) virtually identical to that obtained for the TH–IR fiber staining in layer II of the anterior cingulate cortex of schizophrenia subjects shown in figure 4.

Conclusions

The use of model generation and testing can be very useful means to identify how neural circuitry may be altered in schizophrenia. Without such a strategy, postmortem investigations would essentially use a sophisticated form of "hunting and pecking" to identify patterns of change in complex neuronal networks. The modeling approach provides a means of systematically exploring how small segments of a complex circuit may be altered in schizophrenia. However, model generation and testing is valid only if it is approached in an objective manner, with a strong emphasis on a critical assessment of the data. With this caveat, the principal limitation of this approach is that it only attempts to disprove, rather than prove, a hypothetical construct under investigation. Although this approach probes only a very small portion of much larger circuits, its limited window can nevertheless reveal meaningful changes in the corticolimbic system in patients with schizophrenia. The application of model generation and testing has resulted in a progressive increase in the effect sizes obtained in studies of ACCx–II from schizophrenia patients as the questions asked have become more specific (for example, see Benes et al. 1986 versus Benes et al. 1992*b*). As suggested by Seymour Kety (1959), if one has a hypothesis worth testing and adequate tools to do the testing, it seems reasonable that a critical use of model generation and testing can play an increasingly important role in directing neurobiologists toward meaningful insights into how neural circuitry is altered in schizophrenia.

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